

Dietary copper supplementation reverses hypertrophic cardiomyopathy induced by chronic pressure overload in mice

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Sustained pressure overload causes cardiac hypertrophy and the transition to heart failure. We show here that dietary supplementation with physiologically relevant levels of copper (Cu) reverses preestablished hypertrophic cardiomyopathy caused by pressure overload induced by ascending aortic constriction in a mouse model. The reversal occurs in the continued presence of pressure overload. Sustained pressure overload leads to decreases in cardiac Cu and vascular endothelial growth factor (VEGF) levels along with suppression of myocardial angiogenesis. Cu supplementation replenishes cardiac Cu, increases VEGF, and promotes angiogenesis. Systemic administration of anti-VEGF antibody blunts Cu regression of hypertrophic cardiomyopathy. In cultured human cardiomyocytes, Cu chelation blocks insulin-like growth factor (IGF)-1- or Cu-stimulated VEGF expression, which is relieved by addition of excess Cu. Both IGF-1 and Cu activate hypoxia-inducible factor (HIF)-1 α and HIF-1 α gene silencing blocks IGF-1- or Cu-stimulated VEGF expression. HIF-1 α coimmunoprecipitates with a Cu chaperone for superoxide dismutase-1 (CCS), and gene silencing of CCS, but not superoxide dismutase-1, prevents IGF-1- or Cu-induced HIF-1 α activation and VEGF expression. Therefore, dietary Cu supplementation improves the condition of hypertrophic cardiomyopathy at least in part through CCS-mediated HIF-1 α activation of VEGF expression and angiogenesis.

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Abbreviations used: AAC, ascending aortic constriction; CCO, cytochrome c oxidase; CCS, Cu chaperone for superoxide dismutase-1; Cu, copper; HCM, human cardiac myocyte; HIF, hypoxia-inducible factor; IGF, insulin-like growth factor; RDA, Recommended Dietary Allowance; SOD1, Cu,Zn-SOD; TEPA, tetraethylenepentamine pentahydrochloride; VEGF, vascular endothelial growth factor.

The association of copper (Cu) deficiency with cardiomyopathy and other forms of cardiac disease has been recognized for a long time (1), but its clinical significance has not been fully explored. The current US-Canadian Recommended Dietary Allowance (RDA) for Cu is 0.9 mg/day with a tolerable upper intake level of 10 mg/day for adults 19 years of age or older (2). A debate continues regarding the appropriateness of the RDA for Cu (3). A human study has examined the potential adverse effects of marginal dietary Cu restriction on the cardiovascular system (4). Among 24 subjects consuming a typical American diet (1.03 mg Cu/day), four subjects suffered from cardiac complications after consuming the diet for 4–10 wk. In this case, preexisting cardiac conditions cannot be excluded; however, the fact that these abnormalities appeared after the subjects were fed the controlled low Cu diet does suggest a

detrimental effect of low levels of Cu. Moreover, these cardiac defects disappeared after the affected subjects were supplemented with 3.0 mg Cu/day (4).

An early study of hearts from people who died from ischemic heart disease showed that Cu concentrations were decreased in the uninjured tissues of the infarcted hearts relative to normal heart tissues (5). Another study showed that Cu concentrations decreased in the hearts of people who died from acute ischemic infarction (6). The death rate from ischemic heart disease in soft water areas was found to be increased, and Cu concentrations were lower in the hearts of people who died from chronic ischemic heart disease (7, 8).

Cu supplementation can reverse hypertrophic cardiomyopathy in some patients. One example of this is a rare congenital condition of Sco2 mutations, which affects Cu metabolism and predisposes to hypertrophic cardiomyopathy.

Sco2 is an important Cu chaperon for cytochrome c oxidase (CCO), and mutations in Sco2 result in suppressed CCO activity (9). Patients with mutations in Sco2 developed severe hypertrophic cardiomyopathy (9). A SCO2 patient with severe hypertrophic cardiomyopathy was treated with Cu-histidine. This Cu supplement therapy caused reversal of the hypertrophic cardiomyopathy along with significant improvement in all parameters of heart function and normalization of ECG signs and blood pressure (10). A recent study in a small population of chronic heart failure patients has shown that dietary supplementation with micronutrients for 9 mo increases left ventricle ejection and decreases left ventricle volume along with improvement of quality of life (11). Among the formulated micronutrients in this study was Cu (1.2 mg/day).

To provide a scientific basis for Cu supplementation-induced regression of hypertrophic cardiomyopathy, we used a mouse model of cardiac hypertrophy and dysfunction produced by ascending aortic constriction. In the present study, we found that dietary supplementation with physiologically relevant levels of Cu reverses cardiac hypertrophy and dysfunction even in the presence of chronic pressure overload. The cardiac effect of dietary Cu supplementation is associated with restoration of normal vascular epithelial growth factor (VEGF) expression and enhanced angiogenesis. In the presence of anti-VEGF antibody, dietary Cu supplementation failed to reverse the preestablished heart hypertrophy and dysfunction. We further found that Cu is required for hypoxia-inducible factor (HIF)-1 α transcription activity, which requires the Cu chaperon for superoxide dismutase-1 (CCS) for activation of VEGF expression. These results suggest that dietary Cu supplementation can improve the condition of hypertrophic cardiomyopathy through, at least in part, CCS-mediated activation of HIF-1 α transcription factor for VEGF gene expression.

RESULTS

Regression of cardiac hypertrophy and dysfunction by Cu supplementation

We subjected adult (10–12 wk old) male C57BL/6J mice to chronic pressure overload generated by ascending aortic constriction (AAC) along with sham surgery controls. In mice fed a diet containing RDA adequate levels of Cu (6 mg Cu/kg), cardiac hypertrophy was observed as measured by the increase in heart weight to body weight ratio, by direct observation of the gross anatomy of the heart, and from echocardiography including increases in left ventricle end systolic diameter and posterior wall thickness 4 or 8 wk after AAC surgery (Fig. 1). Cu supplementation (20 mg Cu/kg) during the last 4 wk significantly reversed cardiac hypertrophy (Fig. 1). AAC caused cardiac contractile dysfunction in mice fed 6 mg Cu/kg diet for 4 wk as measured by the percentage of fractional shortening (Fig. 1, %FS) and ejection fraction (Fig. 1, EF) using echocardiography. These mice underwent the transition to heart failure at 8 wk after AAC as indicated by further decreases in %FS and EF and a significant increase in the lung weight to body weight ratio. Cu supplementation completely

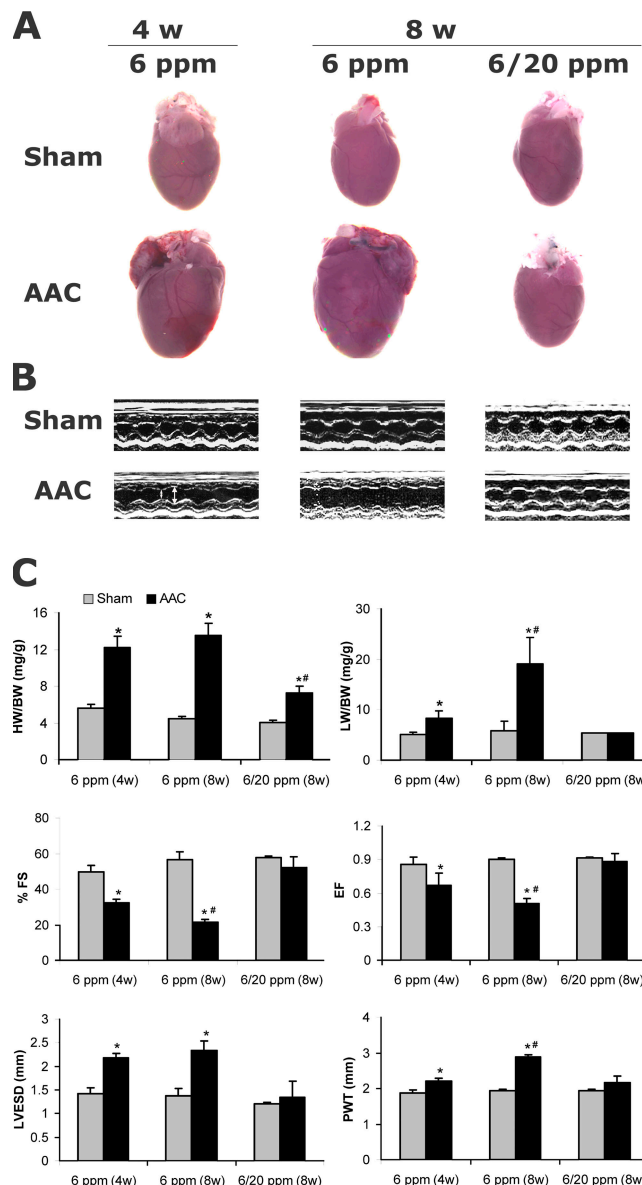


Figure 1. Cu supplementation-induced recovery of cardiac hypertrophy and dysfunction in mice subjected to AAC and fed 6 mg Cu/kg diet (6 ppm). 4 wk after AAC surgery, some mice were kept on the same diet for another 4 wk (8 wks) and others changed to 20 mg/Cu/kg diet (6/20 ppm) for 4 wk. (A) Gross anatomic changes of the hearts. (B) Representative M mode recordings of echocardiography of left ventricles (LV). The LV dimensions at end systole (first vertical line) and at end diastole (second vertical line) were increased at the end of 4 wk after AAC, and further dilation occurred 8 wk after AAC, indicating heart failure. (C) Heart weight to body weight ratio (HW/BW, mg/g); lung weight to body weight ratio (LW/BW, mg/g); the quantitative analysis of the recordings from echocardiograph, including the percentage of fractional shortening (%FS); ejection fraction (EF); left ventricle end-systolic diameter (LVESD); and posterior wall thickness (PWT). *, Significantly different from sham-operated controls ($P < 0.05$, $n = 6-12$). #, Significantly different from AAC-treated mice fed 6 ppm Cu diet for 4 wk ($P < 0.05$, $n = 6-12$).

reversed the contractile dysfunction and prevented the transition to heart failure (Fig. 1).

Histological sections showed cellular hypertrophy along with degenerative morphological changes including necrosis and fibrosis in mice subjected to AAC and fed 6 mg Cu/kg diet for 4 wk, and more severe pathological changes were observed in mice fed the same diet for 8 wk (Fig. 2). Electron microscopic examination revealed mitochondrial abnormalities including swelling, structural disruption, membrane rupture, and the appearance of amorphous matrix densities in mice fed 6 mg Cu/kg diet for 8 wk (Fig. 2). In mice fed 20 mg Cu/kg diet during the last 4 wk after AAC surgery, cardiac pathological changes including necrosis, fibrosis, and ultrastructural abnormalities were all eliminated (Fig. 2). These results thus demonstrate that dietary Cu supplementation reverses preestablished hypertrophic cardiomyopathy induced by chronic pressure overload even in the continued presence of the pathological stimulus.

Cu supplementation restores VEGF levels and promotes angiogenesis in pressure overload hearts

The capillary density in the myocardium subjected to AAC for 4 or 8 wk was significantly decreased in mice fed 6 mg

Cu/kg diet. However, cardiac VEGF levels were significantly increased 4 wk after AAC, but significantly decreased 8 wk after AAC in mice fed 6 mg Cu/kg. Dietary Cu supplementation during the last 4 wk after AAC restored VEGF levels and increased the capillary density in the heart in spite of the continued presence of pressure overload (Fig. 3).

Cu concentrations decrease in hearts subjected to pressure overload

The total levels of Cu in the hearts of mice subjected to AAC and fed 6 mg Cu/kg diet for 4 wk significantly decreased. Dietary Cu supplementation for 4 wk, started at either the same time with AAC surgery or 4 wk after feeding 6 mg Cu/kg diet, effectively prevented or reversed Cu depletion in the heart (Fig. 3). Cu supplementation did not change cardiac Cu levels in the sham controls (Fig. 3). To determine the functional significance of Cu depletion in the heart, the activity of a most sensitive Cu-dependent enzyme complex, cytochrome c oxidase (CCO) was measured. As shown in Fig. 3, CCO activity was significantly depressed 4 wk after AAC and further decreased 8 wk (although not significantly different from 4 wk) after AAC surgery. CCO activity was completely restored 4 wk after Cu supplementation.

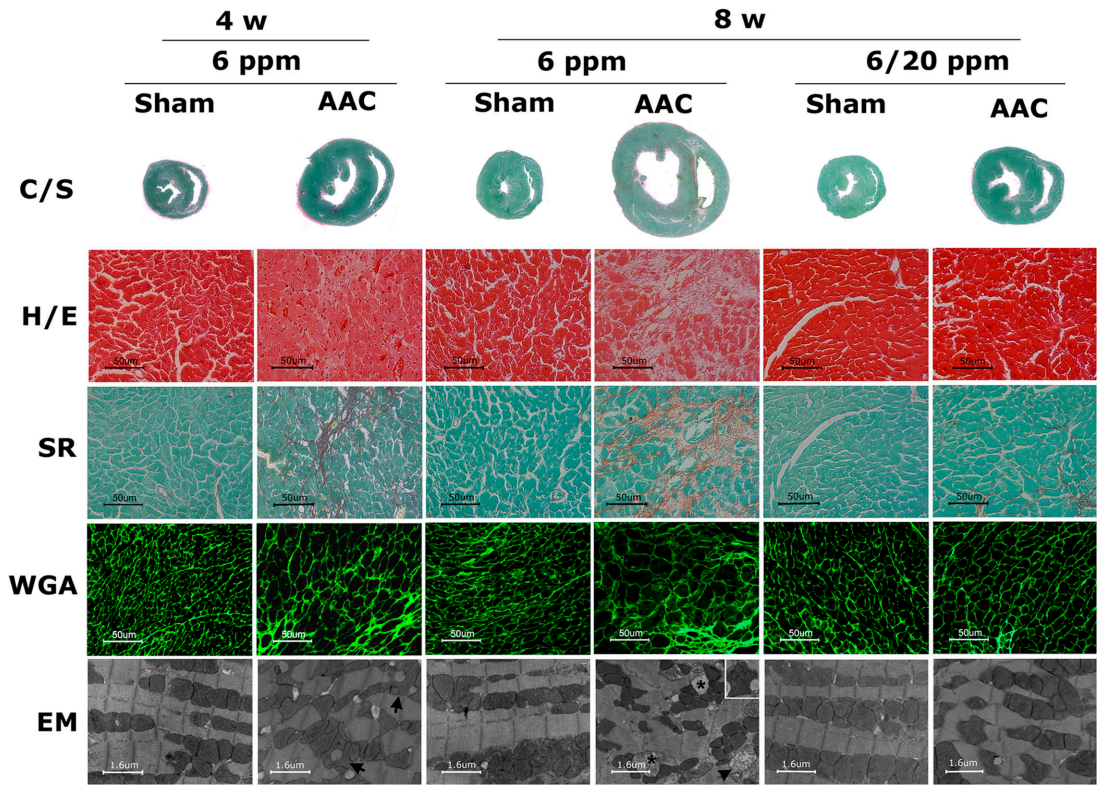


Figure 2. Cu supplementation-induced recovery of morphological abnormalities in mice subjected to AAC and fed 6 ppm Cu diet for 4 wk. Cardiac morphology changes were observed by cross section (C/S), histopathological changes including necrosis (hematoxylin and eosin [H/E]), fibrosis stained by Sirius red (SR), and increased cell size observed by wheat germ agglutinin (WGA) staining for cell membrane. EM revealed

swelling (arrows), structural disruption (asterisks), and membrane rupture (arrowhead) in mitochondria. All of these changes were much more severe at 8 wk but were eliminated by dietary Cu supplementation during the last 4 wk (6/20 ppm) after AAC surgery. Insert in the EM image at 8 wk shows amorphous matrix densities (AMD), a specific severe damage to mitochondria. Bars: 50 μ m (H/E, SR, and WGA panels); 1.5 μ m (EM panels).

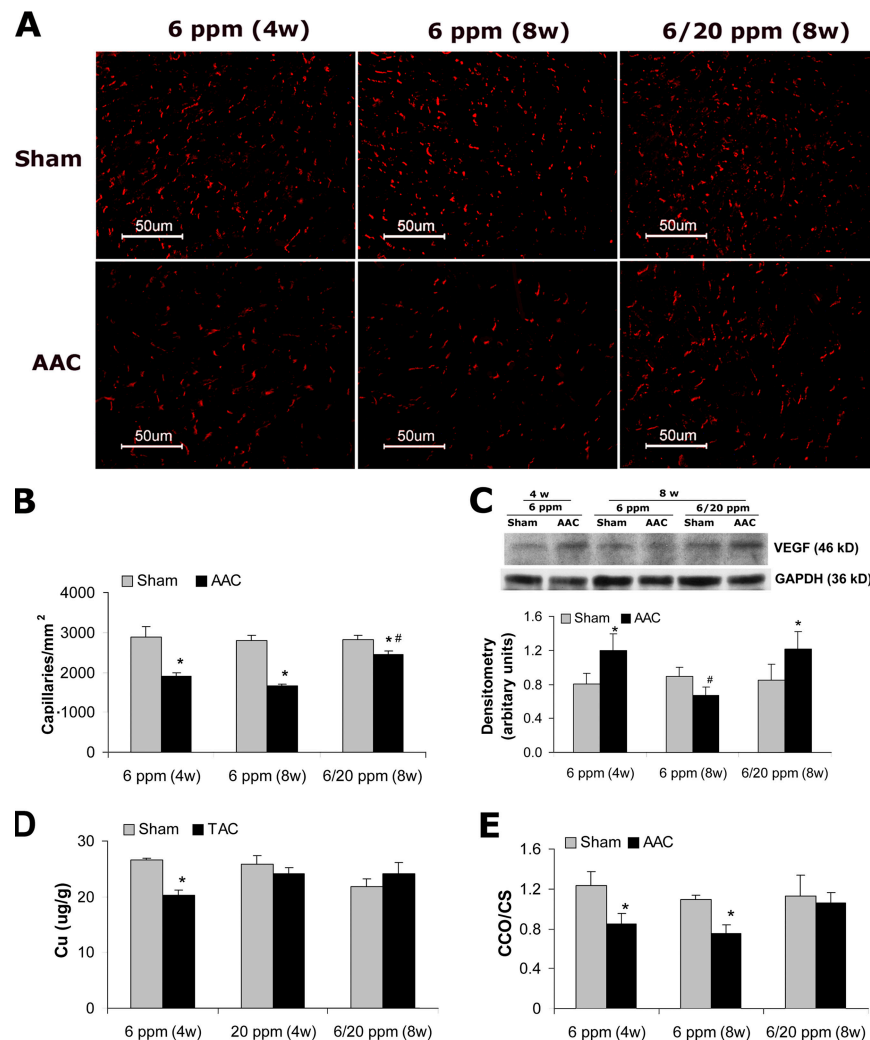


Figure 3. Effect of Cu supplementation on AAC-suppressed VEGF, angiogenesis, and cardiac Cu concentrations. (A) Myocardial capillary density detected by CD31 staining. Bars, 50 μ m. (B) Quantitative analysis of capillary density using a computer-assisted image analyzer (SigmaScan Pro5.0; SPSS, Inc.). Three fields at 200 \times magnification per heart were analyzed and six mice in each group were examined for the calculation. (C) Western blot

analysis of VEGF levels in the heart tissue. (D) AAC-induced decreases in cardiac Cu concentrations 4 wk after AAC and the effect of Cu supplementation. (E) AAC-induced depression of cardiac CCO activity (CCO/CS ratio) 4 or 8 wk after AAC and the effect of Cu supplementation. *, Significantly different from sham-operated controls ($P < 0.05$, $n = 6$). #, Significantly different from AAC-treated mice fed 6 ppm Cu diet for 4 wk ($P < 0.05$, $n = 6$).

Anti-VEGF antibody blunts Cu supplementation-induced regression of hypertrophic cardiomyopathy

We investigated whether or not restoration of myocardial VEGF levels and the associated angiogenesis might be involved in dietary Cu supplementation-induced regression of cardiac hypertrophy and dysfunction by using anti-VEGF antibody. At the time when mice with established cardiac hypertrophy and dysfunction were switched from 6 mg Cu/kg diet to 20 mg Cu/kg diet, they were injected intravenously with anti-VEGF antibody every 3 d for 27 d. This treatment completely blunted Cu supplementation-induced regression of hypertrophic cardiomyopathy. The treatment with anti-VEGF antibody affected neither cardiac morphology nor contractile function of the sham controls (Fig. 4). This result thus suggests that dietary Cu supplementation-induced reversal

of heart hypertrophy and dysfunction is mediated at least in part by restoration of myocardial VEGF expression and the associated angiogenesis.

Cu requirement for HIF-1 α activation of VEGF expression

Because HIF-1 α is a critical transcription factor for VEGF expression, we examined the effect of the Cu chelator, tetraethylenepentamine pentahydrochloride (TEPA), on HIF-1 α transcription activation in cultured human cardiomyocytes. HIF-1 α activation was induced by insulin-like growth factor (IGF)-1, a well-known growth factor stimulating VEGF production by activating HIF-1 α , in comparison with the stimulatory effect of Cu. Both IGF-1 and Cu activated HIF-1 α and stimulated VEGF expression. Importantly, TEPA blocked both the stimulatory effect of IGF-1 and that of Cu (Fig. 5),

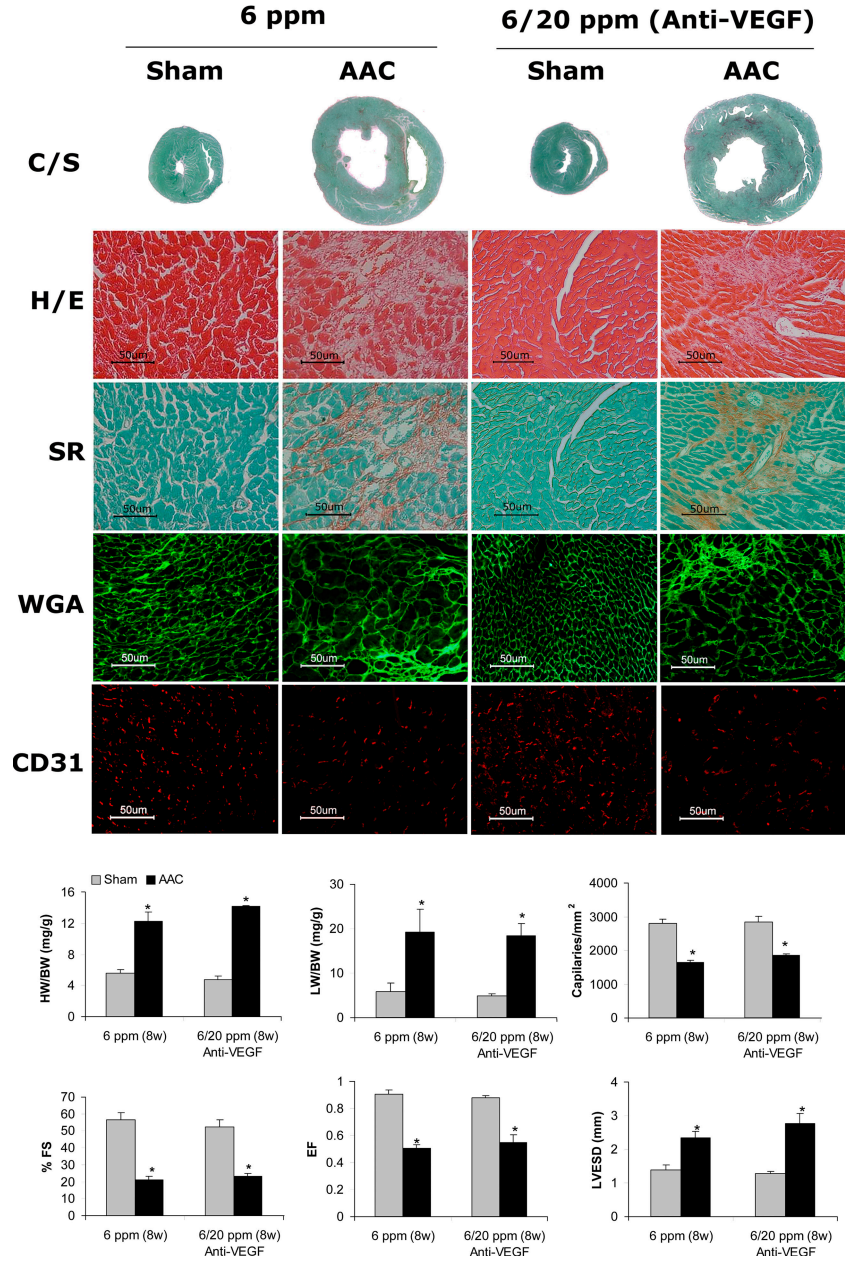


Figure 4. Effect of anti-VEGF antibody administration on Cu supplementation-induced regression of cardiac morphological changes and contractile dysfunction. Mice were fed 6 mg Cu/kg diet (6 ppm) for 4 wk after AAC surgery and then were changed to 20 mg Cu/kg diet for the last 4 wk in the presence of anti-VEGF

antibody administration once every 3 d. All of the parameters and labels are the same as presented in Fig. 1 and 2. *, Significantly different from sham-operated controls ($P < 0.05$, $n = 6$). C/S, cross section; H/E, hematoxylin and eosin; SR, Sirius red; WGA, wheat germ agglutinin. Bars, 50 μ m.

suggesting a requirement for Cu in HIF-1 α activation of VEGF expression. To further test the requirement of Cu for VEGF expression, varying concentrations of CuSO₄ were added into the cultures containing TEPA, and Cu dose dependently relieved TEPA-mediated suppression of VEGF expression (Fig. 6), demonstrating that the inhibitory effect of TEPA acted through Cu chelation. Furthermore, we determined the requirement of HIF-1 α for IGF-1- or Cu-stimulated VEGF expression. We used a siRNA targeting

HIF-1 α and found that in the absence of HIF-1 α , neither Cu nor IGF-1 stimulated VEGF expression in cultured human cardiomyocytes (Fig. 7).

CCS is involved in the interaction between Cu and HIF-1 α

The Cu chaparone CCS is involved in the synthesis of active Cu,Zn-SOD (SOD1) by inserting Cu to the apoprotein. We next examined whether or not Cu activation of HIF-1 α is also mediated by CCS. We used an anti-CCS antibody to

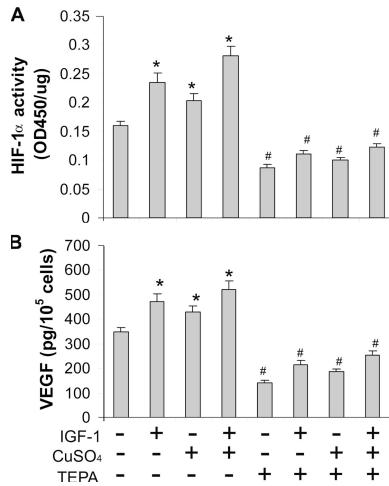


Figure 5. Effect of the Cu chelator TEPA on Cu- and IGF-1-induced HIF-1α activation and VEGF expression and the role of HIF-1α in Cu- and IGF-1-induced VEGF expression in cultured human cardiomyocytes. (A) The effect of TEPA on IGF-1- and Cu-induced HIF-1α transcription activity by ELISA assay. (B) The effect of TEPA on IGF-1- and Cu-induced VEGF expression by ELISA assay. Cells were cultured in the presence or absence of TEPA for 24 h before being exposed to IGF-1 or Cu for 16 h. Values are mean \pm SD ($n = 6$), and the experiment was repeated two times with consistent results. *, Significantly different from untreated controls ($P < 0.05$, $n = 6$). #, Significantly different from corresponding no-TEPA treated cultures ($P < 0.05$, $n = 6$).

perform an immunoprecipitation assay and found that CCS coimmunoprecipitated with HIF-1α (Fig. 8). To explore the role of CCS in HIF-1α activation and VEGF expression, we used a siRNA targeting CCS to delete CCS in cultured human cardiomyocytes. In the absence of CCS, neither Cu nor IGF-1 activated HIF-1α or stimulated VEGF expression (Fig. 8), demonstrating the requirement for CCS in Cu-mediated activation of HIF-1α transcription activity for VEGF expression. Because CCS also transfers Cu to SOD1, we next used a siRNA targeting SOD1 to eliminate the possibility that the Cu effect on VEGF expression is mediated by SOD1. The results, shown in Fig. 9, demonstrate that in the absence

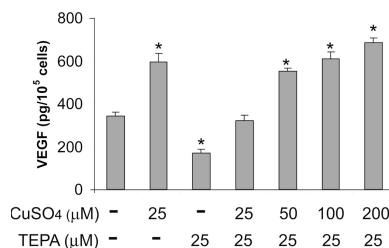


Figure 6. Effect of excess Cu on TEPA-suppressed VEGF expression induced by Cu. Cells were cultured in the presence of 25 μ M TEPA for 24 h before addition of Cu at different concentrations as indicated for 16 h. Values are mean \pm SD ($n = 6$), and the experiment was repeated two times with consistent results. *, Significantly different from untreated controls ($P < 0.05$, $n = 6$).

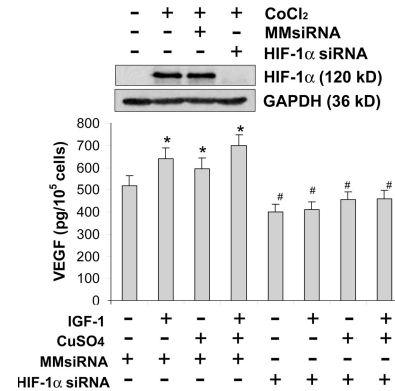


Figure 7. Effects of HIF-1α siRNA on IGF-1- or Cu-induced VEGF expression. Western blot analysis shows that treatment with HIF-1α siRNA for 48 h silenced CoCl₂-induced HIF-1α expression but mismatched siRNA (MMsiRNA) did not. After being treated with the HIF-1α siRNA for 48 h, the cells were treated with IGF-1 or Cu for 16 h and VEGF levels in culture media were analyzed by ELISA assay and normalized by cell numbers. Values are mean \pm SD ($n = 6$), and the experiment was repeated two times with consistent results. *, Significantly different from mismatched RNA only treated (B) controls ($P < 0.05$, $n = 6$). #, Significantly different from corresponding treated cultures ($P < 0.05$, $n = 6$).

of SOD1, Cu- or IGF-1-stimulated VEGF expression was not suppressed, but in contrary, it was further stimulated.

DISCUSSION

In the present study, we report for the first time that Cu supplementation actually reverses hypertrophic cardiomyopathy induced by chronic pressure overload. The results demonstrate that pressure overload generated by AAC causes a decrease in Cu levels in the heart along with hypertrophic cardiomyopathy and the transition to heart failure in mice fed a diet containing RDA adequate levels of Cu. The most important finding is that Cu supplementation to mice with preestablished cardiac hypertrophy and dysfunction in the presence of sustained pressure overload significantly reduced heart hypertrophy and completely restored cardiac contractile function. Cardiac dysfunction is associated with decreased VEGF levels and inhibition of coronary angiogenesis, as demonstrated in previous studies (12,13). Cu supplementation increases VEGF levels and promotes angiogenesis in hypertrophic hearts. VEGF plays a crucial role in the regression, as demonstrated by the observation that anti-VEGF antibody blocked Cu supplementation-induced angiogenesis and regression of hypertrophic cardiomyopathy.

Regression of preestablished hypertrophic cardiomyopathy has been shown recently using the phosphodiesterase-5A inhibitor, sildenafil (14). Our present results indicate that dietary Cu supplementation should be as effective as oral administration of sildenafil in reversing heart hypertrophy and dysfunction induced by chronic pressure overload. However, the simple and inexpensive dietary Cu supplementation would have obvious advantages over sildenafil. In particular, the level of the supplemented Cu is within the physiologic

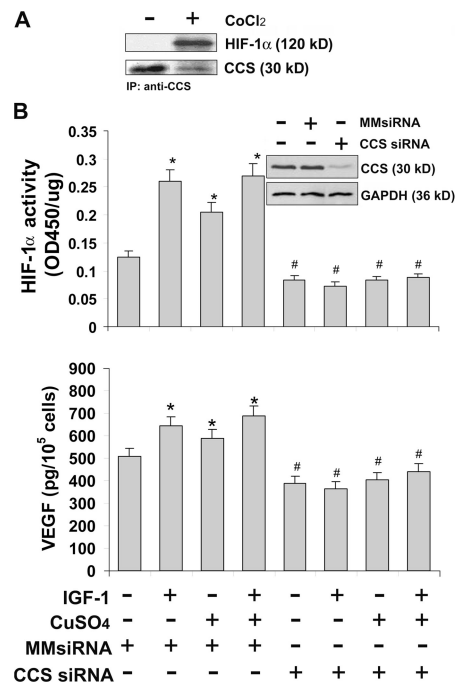


Figure 8. Effect of CCS on Cu- or IGF-1-induced HIF-1α activation and VEGF expression. (A) Coimmunoprecipitation of CCS and HIF-1α. Human cardiomyocytes were treated with CoCl₂ for 16 h to stimulate HIF-1α expression, as indicated. The cell extracts were immunoprecipitated by anti-CCS antibody (Santa Cruz Biotechnology, Inc.) and separated on 10% SDS-PAGE gel and immunoblotted with anti-HIF-1α antibody (Santa Cruz Biotechnology, Inc.) and anti-CCS antibody. (B) Effects of CCS siRNA on IGF-1- or Cu-induced HIF-1α activation and VEGF expression. Western blot analysis shows that treatment of the cells with the CCS siRNA for 48 h silenced the CCS expression but mismatched siRNA (MMsiRNA) did not. After treatment with the CCS siRNA for 48 h, the cells were incubated with IGF-1 or Cu for 16 h. HIF-1α transcription activity was assayed using the cell lysate by an ELISA assay and VEGF concentrations in culture media by an ELISA assay and normalized by cell numbers. CCS siRNA significantly suppressed the transcription activity of HIF-1α and VEGF expression. Values are mean ± SD (*n* = 6), and the experiment was repeated two times with consistent results. *, Significantly different from unstimulated control (*P* < 0.05, *n* = 6). #, Significantly different from corresponding mismatched RNA treated cultures (*P* < 0.05, *n* = 6).

range and well below the maximum tolerable daily intakes (10 mg/day), so that it does not raise safety concerns and should not have the side effects (e.g., headache, flushing, and vision abnormalities) sometimes associated with sildenafil administration. The level of Cu supplementation used in the present study would be equivalent to 2.9 mg/day for humans, which can be found if conventional multiple mineral and vitamin supplements are used (2 mg Cu/day from the supplement plus RDA of 0.9 mg Cu/day).

Our findings are of clinical interest in multiple ways. (a) Given the high prevalence of hypertrophic cardiomyopathy, amelioration of these conditions by simply increasing dietary intakes of Cu would be practical and very attractive. (b) Marginal dietary Cu restriction in humans, defined as consumption

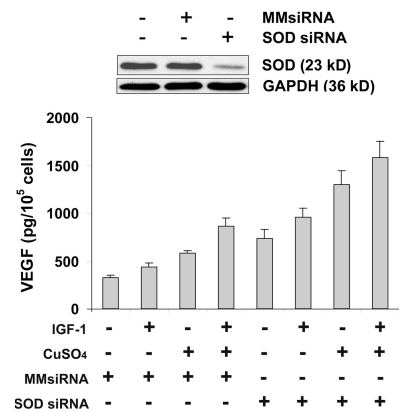


Figure 9. Effects of SOD1 siRNA on IGF-1- or Cu-induced VEGF expression. Treatment with SOD1 siRNA for 48 h silenced the SOD1 expression but mismatched siRNA (MMsiRNA) did not (Western blot). After treatment with the SOD1 siRNA for 48 h, the cells were incubated with IGF-1 or Cu for 16 h. VEGF concentrations in culture media were measured by an ELISA assay and normalized by cell numbers. Values are mean ± SD (*n* = 6), and the experiment was repeated two times with consistent results.

of a diet containing less Cu than the RDA, has been identified from surveys of food consumption (15–17). This would adversely affect those in the population with cardiac complications. Therefore, it appears reasonable to monitor Cu intakes for these populations. (c) The current RDA for Cu has not considered the special case of individuals with cardiac complications. The data obtained here demonstrate that functional deficiency of Cu may occur in these populations and a different RDA for Cu for these populations should be considered. (d) Cu chelation therapy for cancers has recently been in clinical trials (18), but the potential adverse effects of Cu chelation on patients with heart disease need to be evaluated.

An important question is how Cu supplementation causes reversal of hypertrophic cardiomyopathy. We believe that one viable possibility is the effect of Cu on the synthesis of VEGF. A recent study has demonstrated that a lack of coordination between cardiac hypertrophy and angiogenesis (caused by suppression of VEGF activity) is associated with the progression of cardiac hypertrophy to heart failure (12). Acute phase of adaptive cardiac growth is associated with enhanced myocardial VEGF expression, coronary angiogenesis, and preservation of cardiac contractile function. Chronic and pathological hypertrophy is associated with depression of VEGF production and reduced angiogenesis (12). The study has defined that the decrease in VEGF levels is associated with the switching from adaptive to pathological hypertrophy under stress conditions (12). In the present study, we also observed that VEGF production was increased in the early phase, but decreased in the late phase of cardiac hypertrophy induced by sustained pressure overload. However, Cu supplementation restored VEGF production in the late phase of cardiac hypertrophy even in the continued presence of pressure overload.

Cu regulation of VEGF expression has been suggested in several studies (19–22). One study has shown that, at physiologic concentrations, Cu induces the synthesis of VEGF, which promotes angiogenesis in a healing wound (23). In Cu-deficient mouse hearts, generated by dietary Cu restriction, VEGF expression was markedly depressed and Cu repletion of the diet completely restored the expression of VEGF in the heart (22) along with regression of Cu deficiency-induced hypertrophic cardiomyopathy (24). The data presented here further demonstrate the link between Cu and VEGF production and the associated angiogenesis in the heart. Here, we have specifically addressed the question of whether or not VEGF is necessary for the regression of hypertrophic cardiomyopathy. The results clearly show that anti-VEGF antibody-mediated depletion of VEGF prevents Cu supplementation-induced reversal of hypertrophic cardiomyopathy, demonstrating the necessary role of VEGF in the regression.

Is Cu required for VEGF expression in cardiomyocytes? Cu at physiologically relevant levels stimulated VEGF expression, which mimicked the effect of IGF-1 in cultured human cardiomyocytes. The Cu chelator TEPA suppressed not only Cu-stimulated, but also IGF-1-induced VEGF production, indicating that Cu is required for IGF-1-activated VEGF expression. Furthermore, the basal level of VEGF expression was also decreased in the TEPA-treated control cultures, and addition of excess Cu into TEPA treated cultures restored VEGF expression. Therefore, these results strongly indicate that Cu is essential for VEGF expression in cardiomyocytes.

How does Cu regulate VEGF expression? The results showed that HIF-1 α was critically involved in the Cu regulation of VEGF expression. The *in vitro* data that siRNA targeting HIF-1 α abolished Cu stimulation of VEGF expression demonstrate an essential role of HIF-1 α in Cu-dependent VEGF expression in the heart. In the cell, there is virtually no free Cu (25), so that Cu chaperones direct Cu transport specifically to its target molecules. For instance, CCS has been identified as a Cu chaperone for SOD1 (26). CCS interacts directly with SOD1 for the insertion of ionic Cu into the metalloprotein (25). This efficient Cu delivery mechanism avoids inefficient and potentially dangerous trafficking of Cu within the cell. In this context, the interaction between Cu and HIF-1 α could also be mediated by a Cu chaperone such as CCS. In fact, we found that CCS coimmunoprecipitated with HIF-1 α . This is an important novel observation, as it indicates that CCS may not only transfer Cu ion to SOD1 but to other proteins as well. It also indicates that a direct interaction between CCS and the Cu requiring apoproteins may represent a common mechanism for Cu transfer in the cell. However, it is the Cu requiring apoproteins that determine the functional specificity. In this context, we have excluded the possible involvement of SOD1 in Cu regulation of VEGF by showing that SOD1 gene silencing did not inhibit IGF-1- or Cu-stimulated VEGF expression. An unresolved issue is how Cu interacts with HIF-1 α through CCS,

a question that will require more extensive chemical and structural studies.

The result obtained from the use of siRNA targeting CCS demonstrated the importance of CCS in the regulation of Cu-activated HIF-1 α transcription activity and VEGF expression. The inhibitory effect of CCS gene silencing on IGF-1-induced HIF-1 α activation and VEGF expression was as the same as that of Cu chelator, TEPA. Since CCS is a Cu-specific chaperone, the CCS gene silencing result not only provides a mechanistic insight into Cu regulation of VEGF expression but also supports the conclusion obtained from the Cu chelator study for the essentiality of Cu in the regulation of VEGF expression. The CCS gene silencing result also excludes a possible confounding effect of nonspecific metal chelation by TEPA on IGF-1-induced HIF-1 α activation and VEGF expression.

This study for the first time presents evidence that Cu is required for HIF-1 α activation and VEGF expression in cardiomyocytes. Cu activation of HIF-1 α is mediated by CCS, which appears to directly interact with HIF-1 α . The results provide a scientific rationale for Cu supplementation as a means of causing the regression of hypertrophic cardiomyopathy induced by sustained pressure overload. Should similar effects of Cu supplementation be found in controlled studies in human patients, this will point the way to a simple, nontoxic and extraordinarily economical therapy for hypertrophic cardiomyopathy.

MATERIALS AND METHODS

Animal models. Male C57BL/6J mice (10–12 wk old; The Jackson Laboratory) were fed standard AIN-93 diet (27) containing 6 mg Cu/kg, which is the adequate level of Cu for mice according to the RDA established by Food and Nutritional Board of the Institute of Medicine (2) and is equivalent to current RDA for humans (0.9 mg Cu/day). Pressure overload was produced by AAC as described previously (28–30). In brief, mice were anesthetized and ventilated with a rodent respirator. Aortic constriction was made through a left thoracotomy by placing a ligature using a 6-0 silk suture securely around the ascending aorta and a 26 1/2-gauge blunted needle and then removing the needle. For the sham operation, the left thoracotomy was made but the aorta was not ligated. The animal survival rate due to the AAC surgery was 80% including postsurgical death. The animal procedure was approved by the Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation for Laboratory Animal Care.

4 wk after the AAC surgery, some mice were sacrificed for analyses. One group of AAC mice was changed to a diet containing 20 mg Cu/kg, and the other group remained on the 6 mg Cu/kg diet. The supplementation level of Cu in the diet is equivalent to 2.9 mg Cu/day in humans, an intake easily reached if conventional multiple mineral and vitamin supplements are used (2 mg Cu/day plus RDA of 0.9 mg Cu/day). The mice were harvested for the analyses at either 4 or 8 wk after AAC. At the time of harvesting, heart weight to body weight ratio and lung weight to body weight ratio were obtained. Although there were premature deaths of some AAC mice fed 6 mg Cu/kg diet for 8 wk, the number of mice for each parameter measured was six or greater.

Anti-VEGF treatment. Anti-mouse VEGF antibody (R&D Systems, Inc.), 20 μ g in 200 μ l PBS, was given every 3 d by intraperitoneal injection to some mice starting when the mice were switched to the 20 mg Cu/kg diet after feeding 6 mg Cu/kg diet for 4 wk in both AAC surgery and sham controls. The treatment lasted for 27 d.

Echocardiography. Two-dimensional images of the left ventricle were obtained from the apical four- and two-chamber views with a phased-array echocardiography (SONOS 1500 or 2500; Hewlett-Packard, Inc.) and a 2.5-MHz transducer in anesthetized mice. The images were recorded on 1/2-inch videotape for subsequent review and analysis. With the use of a commercially available microcomputer system (GTI; Freeland), the echocardiograms were analyzed qualitatively and quantitatively for the development of regional LV wall motion abnormalities. Quantitative analysis was performed by use of a centerline method that constructs 100 equidistant chords perpendicular to a line centered between digitized LV end-diastolic and end-systolic endocardial borders.

Cu concentrations in the heart. Total Cu concentrations were determined in the heart using inductively coupled argon plasma emission spectroscopy (model 35608; Thermo ARL-VG Elemental) after lyophilization and digestion of the tissues with nitric acid and hydrogen peroxide (31). To confirm the dietary Cu concentrations, a dry-ashing procedure was used. This procedure included dissolution of the residue in aqua regia and measurement by atomic absorption spectrophotometry (model 503; PerkinElmer).

Assessment of morphology changes. Hearts were excised, washed with saline solution, and placed in 10% formalin. Hearts were cut transversely close to the apex to visualize the left and right ventricles. Several sections of heart (4–5- μ m thick) were prepared and stained with hematoxylin and eosin for histopathology or sirius red for collagen deposition and visualized by light microscope. Capillaries were visualized in frozen left ventricle sections stained with antibodies to endothelial cell adhesion molecule-1 (PECAM-1 or CD31) (Santa Cruz Biotechnology Inc.). The capillary density was assessed by a computer-assisted image analyzer (SigmaScan Pro5.0; SPSS, Inc.). Three fields at 200 \times magnification per heart were analyzed, and six mice in each group were examined for the calculation. For examination by electron microscopy, a tissue sample preparation procedure described previously (32) was used.

Cell culture studies. Human cardiac myocytes (HCMs) along with the culture medium were purchased from ScienCell Research Laboratories. The culture medium was composed of basal medium, 5% FBS, 1% cardiac myocyte growth supplement, and 1% penicillin/streptomycin solution (ScienCell). Cells were maintained at 37°C in a 95% humidified air and 5% CO₂. Before each experiment, monolayer HCMs were allowed to grow to 90% confluence, and switched to low-serum medium (basal medium supplemented with 1% FBS) for 24 h, then exposed to 100 ng/ml IGF-1 (Sigma-Aldrich), 25 μ M CuSO₄ (Sigma-Aldrich), or 25 μ M TEPA (Sigma-Aldrich) in the low-serum medium, alone or in combination. The culture media were collected for VEGF measurement, and cells were harvested and subjected to HIF-1 activity assay at 16 h after the treatment.

Gene silencing. siRNAs targeting human HIF-1 α , CCS, and SOD1 and negative mismatched control siRNA were designed and synthesized by Ambion. The siRNA sequences for HIF-1 α were sense, GGCCUCUGUG-AUGAGGCUUtt and antisense, AAGCCUCAUCACAGAGGCCct. The siRNA sequences for CCS, sense, GGACCAGAUGGUCUUGGUAtt, and antisense, UACCAAGACCAUCUGGUCCct. A validated SOD1 siRNA was obtained from the same company (siRNA ID no.: 119429). After the monolayer HCM cultures reached 50% confluence, the cells were transfected with 100 nM annealed siRNAs targeting specific genes or negative mismatched siRNA using a Silencer siRNA transfection kit (Ambion) according to the manufacturer's instruction. At 48 h after the transfection, the culture media were changed to the low-serum medium for 24 h, and then exposed to 100 ng/ml IGF-1 or 25 μ M CuSO₄ alone or in combination. Some cultures were exposed to 150 μ M CoCl₂ (Sigma-Aldrich) to increase HIF-1 α levels. The culture media were collected for VEGF measurement, and cells were harvested and subjected to HIF-1 analysis at 16 h after the last treatment.

HIF-1 activity assay. The nuclear extracts of cultured cells were obtained by using a nuclear extract kit (Active Motif) according to the manufacturer's instruction. HIF-1 activity was quantified in 5–10 μ g of nuclear extracts by specific binding of HIF-1 to an oligonucleotide containing the HRE from the EPO gene (5'-TACGTGCT-3') using a TransAM HIF-1 transcription factor assay kit (Active Motif) following the manufacturer's instruction. Each sample was measured in duplicate, and the measured activity was normalized to total protein content measured by a Bio-Rad Laboratories protein assay kit.

Quantitative analysis of VEGF. VEGF in culture media was measured using a Quantikine Human VEGF Immunoassay (R&D Systems) according to the manufacturer's instruction. Each sample was measured in duplicate, and the VEGF concentrations were normalized by the number of cells in each culture sample.

Enzymatic assay for CCO. Mitochondria were isolated from fresh heart samples using a mitochondrial isolation kit (Pierce Chemical Co.) following the instruction provided. CCO was assayed as described previously (33). In brief, CCO was measured by adding ferrocytochrome c in an isosmotic medium (10 mM KH₂PO₄, pH 6.5, 1 mg/ml bovine albumin, 0.3 M sucrose). The activity was calculated from the rate of decrease in absorbance of ferrocytochrome c at 550 nm ($\epsilon = 19.1 \text{ mM}^{-1}\text{cm}^{-1}$) and normalized by mitochondrial protein content. Because accurate estimations of CCO activity could be compromised by variations in mitochondrial yield and integrity, the CCO activity in the present study was normalized using an invariant marker of mitochondrial enzyme activity, citrate synthase. Citrate synthase was assayed by following the reduction of 5,5'-dithio-bis(2-nitrobenzoic acid) (2 mM) in the presence of acetyl-CoA (0.1 mM) and oxalacetic acid (12 mM) in a medium containing 10 mM KH₂PO₄, pH 7.8, 2 mM EDTA, 1 mg/ml BSA, and 0.1% Triton X-100. The change in absorbance at 412 nm was monitored for 30 min (reflecting the formation of thionitrobenzoate). The citrate synthase activity was calculated from the change in absorbance using $\epsilon = 13.6 \text{ mM}^{-1}\text{cm}^{-1}$, and normalized by mitochondrial protein content.

Immunoprecipitation analysis of CCS and HIF-1 α . HCM cell lysates were prepared in ice-cold IP buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol and protease inhibitor cocktail (Sigma-Aldrich). Crude cell lysates containing 300 μ g protein were pre-cleared by incubating with protein G-Sepharose 4FF (GE Healthcare) and mixed with 4 μ g of rabbit anti-CCS antibody (FL-274; Santa Cruz Biotechnology, Inc.) and incubated at 4°C overnight under rotation. 30 microliters of pre-equilibrated protein G-Sepharose 4FF were then added and incubated for 1 h at 4°C under rotation. After centrifugation at 1,000 *g* for 2 min, supernatants were removed, and the beads were washed three times with the IP buffer. The resulting immunocomplex-containing beads were treated with SDS sample buffer and subjected to Western blot analysis.

Western blot analysis of proteins. The sample preparation from frozen mouse hearts and cultured cells, gel electrophoresis, and transfer to blotting membranes were described previously (34, 35). Nonspecific binding was blocked by using a blocking buffer (5% nonfat dry milk, Tris-buffered saline: 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.5% Tween 20) for 2 h at room temperature, and then the blotting membranes were incubated overnight at 4°C with the following primary antibodies diluted in blocking buffer: rabbit anti-human SOD1 polyclonal antibody (1:1,000; Santa Cruz Biotechnology, Inc.), mouse anti-human HIF-1 α monoclonal antibody (1 μ g/ml; Sigma-Aldrich), goat anti-mouse VEGF polyclonal antibody (2 μ g/ml; R&D Systems), rabbit anti-human CCS (1:2,000; Santa Cruz Biotechnology, Inc.), and mouse anti-GAPDH monoclonal antibody (1:5,000; StressGen Biotechnologies). The membranes were washed in Tris-buffered saline, 1% Tween 20 (TBST) and incubated for 2 h at room temperature with HRP-conjugated rabbit anti-goat IgG and goat anti-mouse or rabbit IgG antibodies (1:20,000; Southern Biotechnology Associates, Inc.) diluted in TBST. The blots were processed and visualized by chemiluminescence (GE Healthcare).

Statistical analysis. Data were expressed as mean \pm SD values and analyzed by ANOVA followed by a Duncan's multiple-range test for further determination of the significance of differences. Differences among groups were considered significant when $P < 0.05$.

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REFERENCES

- Klevay, L.M. 2000. Cardiovascular disease from copper deficiency—a history. *J. Nutr.* 130:489s–492s.
- Trumbo, P., A.A. Yates, S. Schlicker, and M. Poos. 2001. Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. *J. Am. Diet. Assoc.* 101:294–301.
- Klevay, L.M., and D.M. Medeiros. 1996. Deliberations and evaluations of the approaches, endpoints and paradigms for dietary recommendations about copper. *J. Nutr.* 126:S2419–S2426.
- Reiser, S., J.C. Smith Jr., W. Mertz, J.T. Holbrook, D.J. Scholfield, A.S. Powell, W.K. Canfield, and J.J. Canary. 1985. Indices of copper status in humans consuming a typical American diet containing either fructose or starch. *Am. J. Clin. Nutr.* 42:242–251.
- Wester, P.O. 1965. Trace elements in human myocardial infarction determined by neutron activation analysis. *Acta Med. Scand.* 178:765–788.
- Zama, N., and R.L.R. Towns. 1986. Cardiac copper, magnesium, and zinc in recent and old myocardial infarction. *Biol. Trace Elem. Res.* 10:201–208.
- Anderson, T.W., L.C. Neri, G.B. Schreiber, F.D.F. Talbot, and A. Zdrojewski. 1975. Ischemic heart disease, water hardness and myocardial magnesium. *Can. Med. Assoc. J.* 113:199–203.
- Chipperfield, B., and J.R. Chipperfield. 1978. Differences in metal content of the heart muscle in death from ischemic heart disease. *Am. Heart J.* 95:732–737.
- Papadopoulou, L.C., C.M. Sue, M.M. Davidson, K. Tanji, I. Nishino, J.E. Sadlock, S. Krishna, W. Walker, J. Selby, D.M. Glerum, et al. 1999. Fatal infantile cardioencephalomyopathy with COX deficiency and mutations in SCO2, a COX assembly gene. *Nat. Genet.* 23:333–337.
- Freisinger, P., R. Horvath, C. Macmillan, J. Peters, and M. Jaksch. 2004. Reversion of hypertrophic cardiomyopathy in a patient with deficiency of the mitochondrial copper binding protein Sco2: is there a potential effect of copper? *J. Inher. Metab. Dis.* 27:67–79.
- Witte, K.K.A., N.P. Nikitin, A.C. Parker, S. von Haehling, H.D. Volk, S.D. Anker, A.L. Clark, and J.G.F. Cleland. 2005. The effect of micro-nutrient supplementation on quality-of-life and left ventricular function in elderly patients with chronic heart failure. *Eur. Heart J.* 26:2238–2244.
- Shiojima, I., K. Sato, Y. Izumiya, S. Schiekofer, M. Ito, R.L. Liao, W.S. Colucci, and K. Walsh. 2005. Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. *J. Clin. Invest.* 115:2108–2118.
- Izumiya, Y., I. Shiojima, K. Sato, D.B. Sawyer, W.S. Colucci, and K. Walsh. 2006. Vascular endothelial growth factor blockade promotes the transition from compensatory cardiac hypertrophy to failure in response to pressure overload. *Hypertension*. 47:887–893.
- Takimoto, E., H.C. Champion, M.X. Li, D. Belardi, S.X. Ren, E.R. Rodriguez, D. Bedja, K.L. Gabrielson, Y.B. Wang, and D.A. Kass. 2005. Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat. Med.* 11:214–222.
- Klevay, L.M., J.P. Buchet, V.W. Bunker, B.E. Clayton, R.S. Gibson, D.M. Medeiros, P.B. Moser-Veillon, K.Y.T.L.J. Patterson, and W.R. Wolf. 1993. Copper in the western diet (Belgium, Canada, U.K. and USA). In *Trace elements in man and animals—TEMA 8*. M. Anke, D. Meissner, and C.F. Mills, editors. Verlag media touristik, Gersdorf, Germany. 207–210.
- Pang, Y., D.L. MacIntosh, and P.B. Ryant. 2001. A longitudinal investigation of aggregate oral intake of copper. *J. Nutr.* 131:2171–2176.
- Hunt, C.D., and S.L. Meacham. 2001. Aluminum, boron, calcium, copper, iron, magnesium, manganese, molybdenum, phosphorus, potassium, sodium, and zinc: concentrations in common Western foods and estimated daily intakes by infants; toddlers; and male and female adolescents, adults, and seniors in the United States. *J. Am. Diet. Assoc.* 101:1058–1060.
- Pan, Q., C.G. Kleer, K.L. van Golen, J. Irani, K.M. Bottema, C. Bias, M. De Carvalho, E.A. Mesri, D.M. Robins, R.D. Dick, et al. 2002. Copper deficiency induced by tetrathiomolybdate suppresses tumor growth and angiogenesis. *Cancer Res.* 62:4854–4859.
- Ziche, M., J. Jones, and P.M. Gullino. 1982. Role of prostaglandin E1 and copper in angiogenesis. *J. Natl. Cancer Inst.* 69:475–482.
- Mandinov, L., A. Mandinova, S. Kyurkchiev, D. Kyurkchiev, I. Kehayov, V. Kolev, R. Soldi, C. Bagala, E. D. de Muinck, V. Lindner, et al. 2003. Copper chelation represses the vascular response to injury. *Proc. Natl. Acad. Sci. USA.* 100:6700–6705.
- Tapiero, H., D.M. Townsend, and K.D. Tew. 2003. Trace elements in human physiology and pathology. Copper. *Biomed. Pharmacother.* 57:386–398.
- Elsherif, L., Y. Jiang, J.T. Saari, and Y.J. Kang. 2004. Dietary copper restriction-induced changes in myocardial gene expression and the effect of copper repletion. *Exp. Biol. Med. (Maywood)*. 229:616–622.
- Sen, C.K., S. Khanna, M. Venojarvi, P. Trikha, E.C. Ellison, T.K. Hunt, and S. Roy. 2002. Copper-induced vascular endothelial growth factor expression and wound healing. *Am. J. Physiol. Heart Circ. Physiol.* 282:H1821–H1827.
- Elsherif, L., L.P. Wang, J.T. Saari, and Y.J. Kang. 2004. Regression of dietary copper restriction-induced cardiomyopathy by copper repletion in mice. *J. Nutr.* 134:855–860.
- Rae, T.D., P.J. Schmidt, R.A. Pufahl, V.C. Culotta, and T.V. O'Halloran. 1999. Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science*. 284:805–808.
- Culotta, V.C., L.W.J. Klomp, J. Strain, R.L.B. Casareno, B. Krems, and J.D. Gitlin. 1997. The copper chaperone for superoxide dismutase. *J. Biol. Chem.* 272:23469–23472.
- Reeves, P.G., F.H. Nielsen, and G.C. Fahey. 1993. AIN-93 purified diets for laboratory rodents—final report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* 123:1939–1951.
- Ding, B., R.L. Price, T.K. Borg, E.O. Weinberg, P.F. Halloran, and B.H. Lorell. 1999. Pressure overload induces severe hypertrophy in mice treated with cyclosporine, an inhibitor of calcineurin. *Circ. Res.* 84:729–734.
- Shioi, T., J.R. McMullen, O. Tarnavski, K. Converso, M.C. Sherwood, W.J. Manning, and S. Izumo. 2003. Rapamycin attenuates load-induced cardiac hypertrophy in mice. *Circulation*. 107:1664–1670.
- McMullen, J.R., M.C. Sherwood, O. Tarnavski, L. Zhang, A.L. Dorfman, T. Shioi, and S. Izumo. 2004. Inhibition of mTOR signaling with rapamycin regresses established cardiac hypertrophy induced by pressure overload. *Circulation*. 109:3050–3055.
- Chen, Y., J.T. Saari, and Y.J. Kang. 1994. Weak antioxidant defenses make the heart a target for damage in copper-deficient rats. *Free Radic. Biol. Med.* 17:529–536.
- Kang, Y.J., Y. Chen, A. Yu, M. Voss-McCowan, and P.N. Epstein. 1997. Overexpression of metallothionein in the heart of transgenic mice suppresses doxorubicin cardiotoxicity. *J. Clin. Invest.* 100:1501–1506.
- Campian, J.L., M. Qian, X. Gao, and J.W. Eaton. 2004. Oxygen tolerance and coupling of mitochondrial electron transport. *J. Biol. Chem.* 279:46580–46587.
- Feng, W., J. Cai, W.M. Pierce, R.B. Franklin, W. Maret, F.W. Benz, and Y.J. Kang. 2005. Metallothionein transfers zinc to mitochondrial aconitase through a direct interaction in mouse hearts. *Biochem. Biophys. Res. Commun.* 332:853–858.
- Wang, G.W., J.B. Klein, and Y.J. Kang. 2001. Metallothionein inhibits doxorubicin-induced mitochondrial cytochrome c release and caspase-3 activation in cardiomyocytes. *J. Pharmacol. Exp. Ther.* 298:461–468.